A receptor belonging to the integrin superfamily has a heterodimer complex structure in which two subunits, α-chain and β-chain as mutually different membrane proteins are associated with each other non-covalently (Hynes, R. O., Cell, 48, 549-554 (1987)). In the past, the integrin superfamily was classified into three sub-families: β1 integrin, β2 integrin and β3 integrin. Later, new β chains and α chains were discovered one after another, and presently eight β chains (β1,β2,β3,β4,β5,β6,β7 and β8) and fifteen αchains (α1,α2,α3,α4,α5,α6,α7,α8,α9,αv, αL, αM, αX, αIIb and αE) have been identified (Elner, S. G. and Elner, V. M. Inv. Ophtal. Vis. Sci., 37, 696-701 (1996)). It is known that each β chain is associated with one to eight α chains, and as a result, 21 pairs of an α chain and a β chain, i.e., integrin molecules, have been identified (Elner, S. G. and Elner, V. M., Inv. Ophtal, Vis. Sci., 37, 696-701 (1996)). They include α4β1 (VLA-4, β1 integrin),αLβ2 (LFA-1, β2 integrin), αMβ2 (Mac-1, β2 integrin), αIIbβ3 (GPIIb/IIIa, β3 integrin), etc. now targeted for drug development (Drug and Market Development, 6, 201-205 (1995)). Many other integrins are also expected to have relations with diseases.

Please replace the paragraph bridging Pages 3 and 4 with the following:

The heterodimer complex structure of an integrin plays an important role in binding to a ligand (Hynes, R. O., Cell, 48, 549-554 (1987)). For example, it is estimated that the ligand binding region on an integrin consists of both an α chain and a β chain

(Hynes, R. O., Cell, 69, 11-25 (1992)). The fact that integrins having the same α chain but associated with a different β chain, or integrins having the same β chain but associated with a different α chain are respectively different in substrate specificity (Elner, S. G. and Elner, V. M., Inv. Ophtal. Vis. Sci. 37, 696-701 (1996)) supports this assumption. On the other hand, it was reported that the α chains of some integrins have a sequence called an I domain consisting of about 180 amino acids inserted in the molecule, and data suggesting that the I domain only could be bound to a ligand were reported (Ueda, T. et al., Proc. Natl. Acad. Sci. USA, 91, 10680-10684 (1994)). However, it was also reported that the I domain of an α domain and the integrin as its original heterodimer complex are different in the style of binding to a ligand (Kamata, T. and Takada, Y., J. Biol. Chem., 269, 26006-26010 (1994)). It is not clarified yet whether such parameters as specificity and affinity to a ligand are identical. It is not reported in the case of an integrin not containing the I domain, for example, in the case of α4β1, that a partial structure only is bound to a ligand.

D3 CONT

Please replace the paragraph bridging Pages 4 and 5 with the following:

If any integrin isolated and prepared retains its heterodimer complex structure, hence the ligand binding capability, it can be used for studying the style of binding to a ligand in a state close to nature. Furthermore, it can be used as a drug and can also be used as a reagent for measuring the amount of a ligand in tissue or serum or as a material for searching for adhesion inhibiting compounds very usefully. However, isolating and preparing an

D4

integrin with its function retained is said to be very difficult. One reason is that since the association between an α chain and a β chain of an integrin is maintained non-covalently as described before, the chains are easily dissociated during isolation and preparation. Since an integrin is a membrane protein, the necessity of using a surfactant, etc. for solubilization is considered to be a large cause in the dissociation of the complex. In other words, the non-covalent preservation of functional structure inhibits the preparation of such an integrin.

Please replace the paragraph bridging Pages 5 and 6 with the following:

In spite of the difficulty as described above, some cases were reported in which an integrin heterodimer complex was isolated and prepared with its function retained. For cases of α2β1,α5β1 and αvβ3, it was reported that the binding to a ligand can be determined by letting a liposome incorporate an integrin purified by using affinity column chromatography (Santoro, S. A. et al., Biochem. Biophys. Res. Comm., 153, 217-223 (1988), Pytela, R. et al., Cell, 40, 191-198 (1985), Pytela, R. et al., Method Enzymol., 144, 475-489 (1987)). For other cases, it was reported that if purified α5β1 or αvβ3 is coated on a plate, a peptide which inhibits the cell adhesion through the integrin can be selected (Koivunen, E. et al., J. Biol. Chem., 268, 20205-20210 (1993), Healy, J. M. et al., Biochemistry, 34, 3948-3955 (1995)). For further other cases,

it was reported that if purified αvβ3 or α4β1 coated on a plate, the binding to a ligand can be determined (Charo, I. F. et al., J. Cell Biol., III, 2795-2800 (1990), Makarem, R. et al., J. Biol. Chem., 269, 4005-4011 (1994), Paul Mould, A. et al., J. Biol. Chem., 269, 27224-27230 (1994)). For a still further other case, it was reported that if an extracellular portion of αIIbβ3 heterodimer complex prepared by gene manipulation is coated on a plate through a complex specific antibody, the binding to a ligand can be determined (Gulino, D. et al., Eur. J. Biochem., 227, 108-115 (1995)). These cases suggest that to exert the function of a purified integrin, its heterodimer complex must be bound to or included in a carrier. The reason a carrier is considered to be necessary is that since a heterodimer complex is associated non-covalently in a solution, it tends to dissociate, and as a result, cannot retain its functional structure. In the finally stated case, only a molecule with a heterodimer complex structure is selected using a complex specific antibody, in a design to determine the binding in a state where both the chains are not dissociated from each other.

D5 cont

Please replace the paragraph bridging Pages 6 and 7 with the following:

As a case requiring no carrier, it was reported that purified α1β1, or α2β1 allows the determination of the bonding to a ligand dependent on high concentration of metal ions even without using any carrier (Pfaff, M. et al., Eur. J. Biochem., 225, 975-984

D6

(1994)). In this case, the surfactant added in the process of purification plays a role similar to that of a liposome, acting as a carrier. For a further other case, it was reported that an extracellular $\alpha M\beta^2$ heterodimer complex prepared by using gene manipulation is bound to a ligand (Berman, P. W. et al., J. Cell Biochem., 52, 183-195 (1993)). These cases do not suggest the necessity of any carrier as described before, but the disadvantage that the association of molecules in a heterodimer complex is retained non-covalently is not improved.

D6 cont

Please replace the paragraph bridging Pages 7 and 8 with the following:

Of the molecules belonging to the integrin superfamily, integrin $\alpha 2\beta 1$ is an extracellular matrix receptor expressed in T cells, platelets, etc. activated for a long time. However, it was reported that the $\alpha 2\beta 1$ on the cell surfaces of platelets and fibroblasts is bound to collagens only and that the $\alpha 2\beta 1$ on the surfaces of vascular endothelial cells is bound to both collagens and laminins (Elices, M. J. et al., Proc. Natl. Acad. Sci. USA, 86, 9906-9910 (1989)), and it is speculated that the function of $\alpha 2\beta 1$ becomes different, depending on cell type.

D7

Please replace the first full paragraph on Page 8 with the following:

In relation to the conditions of diseases, there are reports to suggest that integrin $\alpha 2\beta 1$ plays an important role for wound healing and cancerous metastasis (Shiro, J. A. et al., Cell, 67,

D8

403-410 (1991), Chen, F. et al., J. Exp. Med., 173, 1111-1119 (1991), Chan, B. M. C. et al., Science, 251, 1600-1602 (1991)). Furthermore, it was reported that from the analysis of platelet function of patients with bleeding tendency, the adherence of platelets and collagens through integrin α2β1 has close relation with the first step of hemostasis and thrombosis process (Nieuwenhuis, H. K. et al., Nature, 318, 470-472 (1985)). Though the relations of integrin α2β1 with conditions of diseases are suggested like this, any medical application of using the integrin α2β1 protein and other isolated extracellular matrix receptor proteins under physiological ion conditions or in the presence of plasma components has not been examined.

D8 Cont

Please replace the paragraph bridging Pages 8 and 9 with the following:

The present invention relates to chimeric proteins in which the α chain and β chain of an integrin are combined with the heavy chain or light chain of an immunoglobulin, their heterodimer complexes, a production process thereof, a method for testing the binding of an integrin-immunoglobulin chimeric protein heterodimer complex to a ligand and a cell, substances bound to an integrin obtained by using the method, a method for searching for a substance inhibiting the binding between an integrin and a ligand using the integrin-immunogobulin chimeric protein heterodimer complex, substances for inhibiting the binding, and the application

D9

of integrin-immunoglobulin chimeric protein heterodimer complexes as drugs and reagents. Furthermore, the present invention relates to platelet substitutes containing an integrin-immunoglobulin chimeric protein heterodimer complex or any other isolated extracellular matrix receptor as an active ingredient.

Please replace the first full paragraph on Page 9 with the following:

Fig. 1 shows that α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex is bound to VCAM-1 expressing cells, and that the binding is inhibited by an anti-integrin antibody or EDTA, a cationic chelating agent.

Please replace the paragraph bridging Pages 9 and 10 with the following:

Fig. 3 shows that the binding between α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex and CS-1 peptide is inhibited by the peptide GPEILDVPST (SEQ ID NO.:16), and is not inhibited by the peptides GPEILDVPST (SEQ ID NO.:17) or GRGDSP (SEQ ID NO.:18).

Please replace the paragraph bridging Pages 12 and 13 with the following:

In the present invention, a molecule in which a chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of the β chain of the integrin and the heavy chain or

light chain of the immunoglobulin are associated with each other is called an integrin-immunoglobulin chimeric protein heterodimer complex. In this case, a combination consisting of α chain•immunoglobulin heavy chain (which means a chimeric protein consisting of an α chain and the heavy chain of an immunoglobulin; hereinafter this applies) and β chain •immunoglobulin heavy chain, a combination consisting of α chain• immunoglobulin heavy chain and β chain•immunoglobulin light chain, and a combination consisting of α chain• immunoglobulin light chain and β chain•immunoglobulin heavy chain are preferable. A combination consisting of α chain•immunoglobulin heavy chain and β chain•immunoglobulin heavy chain is more preferable.

D2 Cont

Please replace the first full paragraph on Page 13 with the following:

In the integrin-immunoglobulin chimeric protein heterodimer complex of the present invention, the α chain can be α1,α2,α3,α4,α5,α6,α7,α8,α9,αv, αL, αM, αX, αIIb or αE, and the β chain can be β1,β2,β3,β4,β5,β6,β7 or β8. It is preferable that the α chain is α4 or α2 and that the β chain is β1, though preferable chains are not limited to them.

D3

Please replace the second full paragraph on Page 13 with the following:

The process for preparing an integrin- immunoglobulin chimeric protein heterodimer complex is described below, but the process is not limited thereto.

D4

Please replace the paragraph bridging Pages 14 and 15 with the following:

Then, a DNA coding for an immunoglobulin is prepared. In the present invention, it is desirable to use DNAs coding for the heavy chain and light chain of a human immunoglobulin, but DNAs coding for an immunoglobulin of another animal species can also be used. The preparation of a DNA coding for human IgG is already reported (Ellison, J. W. et al., Nucleic Acids Res., 10, 4071-4079 (1982)), but the preparation is not limited to this method. Any method similar to the above mentioned method for preparing DNAs coding for the α chain and β chain of an integrin can also be used. In the present invention, for the heavy chain of a human immunoglobulin, it is preferable to use a genomic DNA, but a cDNA can also be used. As the DNA for the heavy chain of a human immunoglobulin, it is preferable to use a portion coding for the hinge region, CH2 region or CH3 region, but a DNA coding for the entire constant region of CH1-CH3 can also be used. For the light chain of an immunoglobulin, a DNA coding for the CL region is used. Finally, a DNA coding for the extracellular portion of an α chain or β chain and a DNA coding for the constant region of human immunoglobulin heavy chain are linked in frame. The obtained DNA codes for a polypeptide starting from the methionine of translation initiation and having the signal sequence of the α chain or β chain of an integrin, its extracellular region and the constant region of human immunoglobulin heavy chain linked in this order.

D16

Please replace the first full paragraph on Page 16 with the following:

The DNA coding for a chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin, or the DNA coding for a chimeric protein consisting of the β chain of an integrin and the heavy chain or light chain of an immunoglobulin respectively obtained in the above is functionally linked in a proper expression control sequence, to obtain a recombinant vector. The general methods concerning gene recombination such as the method for preparing the recombinant vector, the method for transfecting it into a cell are described in a published book ("Molecular Cloning", Sambrook et al., (1989) Cold Spring Harbor Lab. Press, New York), but the methods are not limited to those stated there. In the present invention, it is desirable to use an expression control sequence suitable for protein expression in animal cells. For example, for expression of insect cells, polyhedrin promoter, p10 promoter, etc. are generally used as expression control sequences, and for expression of other animals' cells, SRα promoter, cytomegalovirus derived promoter, simian virus 40 derived promoter, polyhedrin promoter, p10 promoter, etc. are used. However, the expression control sequences are not limited to them. In the present invention, it is preferable to use SRα promoter.

D17

Please replace the paragraph bridging Pages 17 and 18 with the

following:

It is known that the methods for transfecting a recombinant vector into a cell include the lipofectin method, calcium phosphate method, electroporation method, etc., and any of the methods can be used. The method is not limited to them. It is preferable that when a cell is transfected by using a recombinant vector, a recombinant vector for expression of a chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin and a recombinant vector for expression of a chimeric protein consisting of the β chain of the integrin and the heavy chain or light chain of the immunoglobulin are transfected into the cell one after another using different drug resistance markers. The recombinant vectors can be transfected in any order or simultaneously. It is desirable that the two recombinant vectors to be transfected are vectors for expression of a combination consisting of α chain•immunoglobulin heavy chain (which means a chimeric protein consisting of an α chain and the heavy chain of an immunoglobulin; hereinafter this applies) and β chain• immunoglobulin heavy chain, or α chain•immunoglobulin heavy chain and β chain•mmunoglobulin light chain, or α chain• immunoglobulin light chain and β chain•immunoglobulin heavy chain. Any of these combinations can be adopted, but a combination of recombinant vectors for expression of α chain•immunoglobulin heavy chain and β chain•immunoglobulin heavy chain is desirable.

D18

Please replace the paragraph bridging Pages 18 and 19 with the following:

In any transfection method and any combination of vectors, it is important to select a cell which is transfected by the two recombinant vectors and produces a chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin simultaneously almost by the same amounts. This can be achieved by measuring the amounts of the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin produced in the cultured supernatant solution of the cell transfected by the recombinant vectors. For measurement, for example, the transfected cell can be cultured in a medium containing ^{35}S according to any publicly known method, for labeling the proteins, and the amounts of the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin existing in the cultured supernatant solution can be estimated by immunoprecipitation using an anti-α chain antibody or an anti-β chain antibody respectively. As another method, the amounts of the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and

D19

the heavy chain or light chain of an immunoglobulin existing in the cultured supernatant solution can be estimated according to the ELISA method using an anti-human immunoglobulin antibody and an anti-α chain antibody or an anti-β chain antibody. Anyway, it is preferable to select a clone which produces almost the same large amounts of the chimeric proteins of the α and β chains in the cultured supernatant solution, for preparing an integrin-immunoglobulin chimeric protein heterodimer complex. The methods for labeling proteins, the methods of immunoprecipitation and the general methods of ELISA are described in a published book ("Antibody" Harlow, E., and Lane, D. (1988), Cold Spring Harbor Lab. Press, New York), but the methods are not limited to them. Any other method can also be used for detecting chimeric proteins.

D19 Cont

Please replace the paragraph bridging Pages 21 to 23 with the following:

A purified integrin-immunoglobulin chimeric protein heterodimer complex can be identified as a protein showing at least one band under non-reducing conditions and at least two bands under reducing conditions by SDS-PAGE. SDS-PAGE also can be used to confirmthat the heterodimer is linked by the disulfide bond between immunoglobulin heavy chains. It sometimes occurs that a plurality of bands are detected under reduction, but this is considered to be probably because intramolecular cleavage of the α chain has occurred. Especially, with α4, this phenomenon is known (Hemler, M.E. et al., J. Biol. Chem., 262, 11478-11485 (1987)).

D20

Furthermore, it can be confirmed by the Western blotting method that the respective bands indicate chimeric proteins. As another method, it can be confirmed by the ELISA method combining an anti-α chain antibody, anti-β chain antibody and anti-human immunoglobulin antibody, that the obtained molecule is an integrin-immunoglobulin chimeric protein heterodimer complex. That is, the molecule can be identified as a protein molecule with epitopes for all the antibodies. As a further other method, an integrin-immunoglobulin chimeric protein heterodimer complex can also be identified by immunoprecipitation. In this case, if the purified protein is labeled by ^{35}S, or ^{125}I or biotin, etc. according to any known method, and immunoprecipitated using an anti-αchain antibody, anti-β chain antibody and anti-human immunoglobulin antibody, the same electrophoretic pattern can be obtained in every case. So, it can be confirmed that the integrin-immunoglobulin chimeric protein heterodimer complex has the intended structure. Furthermore, even if a condition to dissociate the integrin complex on a cell membrane such as the coexistence of EDTA or boiling in the presence of SDS is applied, the immunoprecipitation pattern is not changed. So, it can be confirmed that the obtained integrin-immunoglobulin chimeric protein heterodimer complex is a structurally stabilized complex. The methods for confirming an integrin-immunoglobulin chimeric protein heterodimer complex are not limited to those stated above.

Please replace the paragraph bridging Pages 23 and 24 with the following:

The binding between a prepared integrin-immunoglobulin chimeric protein heterodimer complex and a ligand can be tested as described below. After a ligant and an integrin-immunoglobulin chimeric protein heterodimer complex are brought into contact with each other, to make a mixture, the amount of the integrin-immunoglobulin chimeric protein heterodimer complex bound to the ligand or the amount of the ligand bound to the integrin-immunoglobulin chimeric protein heterodimer complex is measured. The amount of an integrin-immunoglobulin chimeric protein heterodimer complex can be measured by labeling the complex itself by a fluorescent dye or enzyme or radioisotope, etc. The amount of a ligand can also be measured by any similar method. A detection method such as SPA (Amersham) can also be used for the measurement. Furthermore, any reagent which can recognize a complex or ligand labeled by a fluorescent dye, enzyme or radioisotope, etc. can also be used for the measurement. The reagent for recognizing an integrin-immunoglobulin chimeric protein heterodimer complex can, for example, be an anti-human immunoglobulin antibody. In this test, it is preferable to bind the molecule to be detected, to any carrier such as a bead or plate. As a ligand, its entire molecule can be used, but a portion retaining the binding activity to an integrin can also be taken out for use. For example, for integrin α4β1 or integrin α2β1, its

D21 Cont ligand, fibronectin or collagen or its peptide fragment bound to a carrier can also be used.

Please replace the paragraph bridging Pages 25 and 26 with the following:

A case where a purified integrin was coated on a plate to search for a peptide to be bound was reported (Healy, J. M. et al., Biochemistry 34, 3948-3955 (1995)). Even if the integrin-immunoglobulin chimeric protein heterodimer complex obtained in the present invention is used, a substance to be bound to an integrin can be similarly searched for. Especially when the chimeric protein heterodimer complex of the present invention is used, the operation to remove the non-specifically bound substances can be effected under more severe conditions. So, the operation can be D22 simplified advantageously. Furthermore, since the complex is not dissociated during operation, a bound substance can be selected more specifically advantageously. Known sources suitable for selecting bound substances include a phage peptide library (e.g., Scott, J. K. and Smith, G. P., Science, 249, 386-390 (1990)) and a DNA oligomer library (e.g., O'Connel, D. et al., Proc. Natl. Acad. Sci. USA, 93, 5883-5887 (1996)), but in the present invention, it is preferable to use the former.

Please replace the paragraph bridging Pages 26 and 27 with the following:

D23 An extracellular matrix receptor preferably used as a platelet substitute is an integrin. The α chain of the integrin can be

α1,α2,α3,α4,α5,α6,α7,α8,α9,αv,αL,αM, αX,αIIb or αE, and among them, α2 is preferable. The β chain can be β1,β2,β3,β4,β5,β6,β7or β8, and among them, β1 is preferable. Integrin α2β1 is more preferable. The receptor source for isolation can be a tissue or cell expressing an extracellular matrix receptor, or a dissolved membrane fraction of a receptor expressing cell prepared by gene manipulation, etc. It is more preferable to design for obtaining a soluble protein by modifying a receptor gene by gene manipulation, and to use the cultured supernatant solution of the cells capable of producing it, as a source. Furthermore in the design of the soluble protein, it is preferable that the functional structure of the extracellular matrix receptor is retained. For example, it is desirable to use an integrin-immunoglobulin chimeric protein heterodimer complex obtained by modifying the heterodimer structure of an integrin to allow its α and β chains to be covalently associated with each other. As the integrin-immunoglobulin chimeric protein heterodimer complex, it is preferable that the α chain of the integrin is α1,α2,α3,α4,α5,α6,α7,α8,α9,αv,αL,αM,αX,αIIb or αE, and among them, α2 is more preferable. Furthermore, it is preferable that the β chain is β1,β2,β3,β4,β5,β6,β7 or β8,and among them, β1 is more preferable. It is further more preferable that the α chain is α2 and that the β chain is β1. The platelet substitute of the present invention is described below mainly in reference to a typical extracellular matrix receptor, integrin α2β1- immunoglobulin chimeric protein

D23 Cont

heterodimer complex, but the present invention is not limited thereto or thereby.

Please replace the first full paragraph on Page 28 with the following:

For use as a platelet substitute, it is preferable to bind an integrin α2β1-immunoglobulin chimeric protein heterodimer complex to a liposome covalently according to the method stated in a report (Martin, F. J. et al., Biochemistry, 20, 4229 (1981)). The carrier can also be any other drug carrier than a liposome as far as its use for drugs is permitted. If a liposome is used as the carrier, the liposome is prepared according to the composition and method stated in a published book "Preparation and Experiments of Liposomes (in Japanese)", Oku, N. (1994), Hirokawa Shoten), but a preferable method is such that the epitope bound to the extracellular matrix of an integrin α2β1-immunogloblulin chimeric protein heterodimer complex is exposed outside the liposome membrane.

Please replace the paragraph bridging Pages 28 to 29 with the following:

For confirming that an integrin α2β1-immunoglobulin chimeric protein heterodimer complex is bound on the prepared liposome carrier, a flow cytometer is used. The reagents which can be used for recognizing the integrin α2β1-immunoglobulin chimeric protein heterodimer complex include an anti-integrin α2 antibody, anti-integrin β1 antibody, anti-human immunoglobulin antibody, etc. If

the antibody used is fluorescently labeled, it can be used for determination directly, but if it is not fluorescently labeled, a secondary antibody which recognizes the immunoglobulin class of the animal species used for preparing the antibody is used as a fluorescent label. As a further other confirmation method, the integrin α2β1-immunoglobulin chimeric protein heterodimer complex itself can be labeled by an enzyme or radioisotope, etc., for confirmation in proper combination with a color dye or radioactivity measuring instrument, etc.

D25 cont

Please replace the paragraph bridging Pages 29 and 30 with the following:

To examine the extracellular matrix binding capability using an integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome, it is preferable to suspend the integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome into a buffer with a physiological cation concentration or plasma. The buffer with a physiological cation concentration refers to a buffer containing at least cations such as Mg ions or Ca ions and adjusted to about neutrality. The plasma is prepared by processing the blood collected in the presence of an anticoagulant, according to a general plasma preparation method. As the anticoagulant, for example, heparin or EDTA solution can be added by sufficient units. A marketed normal plasma, coagulation factor deficient plasma or serum, etc. can also be used. However, if the anticoagulant used lowers the cation concentration, cations are added to achieve a

D26

physiological concentration later. Then, the integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome is mixed with an extracellular matrix or its fragment coated on a carrier for a certain time, to judge whether binding takes place. It is preferable that the coating of the extracellular matrix or its fragment as a solid phase is achieved by using a plastic plate, etc., but marketed beads for forming an extracellular matrix as a solid phase, etc. can also be used. When a collagen is used as the extracellular matrix, any animal species and type can be used. The binding reaction between an integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome and an extracellular matrix is effected according to a general method adopted for observing the adherence reaction of platelets. In many cases, they are allowed to stand mainly in a static system for a certain time, to induce binding to the matrix, but it is preferable to apply a shaking load or shear stress, etc.

D26 Cont

Please replace the paragraph bridging Pages 30 and 31 with the following:

The integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome is bound to an extracellular matrix under the conditions as described above, and the amount of binding is measured by applying the above mentioned ELISA method using an anti-human immunoglobulin antibody. For more accurate determination, it is desirable to immobilize the liposome bound to the matrix by 1% glutaraldehyde, etc. As an alternative method to

D27

the ELISA method, for example, if a radio-labeled lipid is incorporated into the liposome beforehand, the amount of the liposome bound to the extracellular matrix can be obtained as radioactivity. Furthermore, to qualitatively judge the binding and covering degree to the extracellular matrix, a labeled antibody for recognizing the integrin α2β1-immunoglobulin chimeric protein heterodimer complex on the bound liposome can be combined with a color dye, etc., to dye the portions where the liposome is bound. It is more preferable that the generally used tissue antibody dyeing method is used to use a peroxidase labeled antibody against the integrin α2β1- immunoglobulin chimeric protein heterodimer complex and diaminobenzidine in combination, but the measuring method is not limited to it. As a further alternative method, the area covering the extracellular matrix can be obtained as a covering rate using an image processing analyzer.

D27 CONT

Please replace the paragraph bridging Pages 31 and 32 with the following:

Methods for examining the hemostasis of platelets include testing the adhering capability of platelets to the extracellular matrix and the agglutination capability induced by a collagen ("Handbook on the Examination of Blood Coagulation (in Japanese)", p. 65-78, Fukutake, M. and Fujimaki, M. (1987), Uchudo Yagi Shoten, Santro, S.A., Cell, 46, 913-920 (1986), Lethagen, S. and Rugarrn, P., Thrombo Haemost., 67, 185-186 (1982)). Especially the adhering capability of platelets to the extracellular matrix is an indicator

D28

of primary hemostasis. The adhering capability is evaluated by using blood as it is, or platelet rich plasma or platelets washed by a buffer with physiological ions. Therefore, whether or not the integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome obtained in the present invention can be a functional substitute of platelets can be judged in reference to its binding capability and the level of the binding capability to the extracellular matrix in the existence of plasma components or at a physiological ion concentration.

D28 Cont

Please replace the first full paragraph on Page 32 with the following:

If the binding capability of the integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome obtained in the present invention to the extracellular matrix in the presence of the plasma components is strong, it suggests that the liposome can be a platelet substitute. Therefore, it can be used as a therapeutic or preventive agent against the congenital and acquired bleeding tendency due to platelet abnormality, and also widely as a platelet transfusion substitute.

D29

Please replace the paragraph bridging Pages 32 and 33 with the following:

Similarly the integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome obtained in the present invention can be a therapeutic or preventive agent for conditions of diseases where vascular endothelial cell disorder is a problem. For

D30

example, it was reported that in the prognosis of PTCA (percutaneous coronary restenosis), the excessive accumulation of platelets on the extracellular matrix exposed by balloon catheter treatment triggers restenosis (Liu, M.W. et al., Circulation, 79, 1374-1378 (1989)). In Example 22, the effect of the integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome to cover the extracellular matrix was confirmed, and this effect can reduce the excessive accumulation of platelets to allow use also as a restenosis preventive treatment. Furthermore, if the integrin α2β1-immunoglolublin chimeric protein heterodimer complex liposome is labeled by a medically allowable method, it can be used for monitoring the region of the extracellular matrix exposed by vascular endothelial cell injury, and furthermore, if a drug is enclosed in the liposome, it can also be applied to the targeting therapy for a local injured region.

Please replace the paragraph bridging Pages 33 and 34 with the following:

When any integrin α2β1-immunoglobulin chimeric protein heterodimer complex liposome stated in the present invention is used as a platelet substitute, the administration paths include infusion, intravenous administration, etc., and it is usually used by being suspended in any physiologically suitable solution such as a salt solution or plasma, etc. It can be used alone or also in combination with another chimeric protein heterodimer complex with an extracellular matrix receptor or its immunoglobulin. It can

also be used together with another drug containing total platelets. The dose is properly selected to suit the symptom, age, body weight, etc., and can be 0.1 mg to 10 g per day as the amount of the protein for an adult, being able to be administered at a time or in several times. It can also be mixed with a pharmaceutically allowed carrier or excipient, etc., to be applied locally to the injured region as an externally applied drug such as an ointment, liniment or plaster. In this case, the externally applied drug is prepared to be 1 ng/cm^2 to 1 mg/cm^2 as the amount of the protein per one time of coating.

D31 Cont

Please replace the paragraph bridging Pages 34 and 35 with the following:

As human IgG_1 genome gene, a clone identical with reported base sequence information (Ellison, J. W. et al., Nucleic Acids Res., 10, 4071-4079 (1982)) was acquired from a human genomic library (CLONTECH) using a hybridization cDNA probe based on the sequence information. This was used as the template DNA for PCR. As primers for amplifying the DNA fragment containing the hinge region (H) and the constant region portions (CH2 and CH3) of human IgG_1 gene, a DNA oligomer shown in SEQ ID NO:4 of the sequence listing (hereinafter a sequence No. of the sequence table is simply called a SEQ ID NO:) with BamHI restriction site and a DNA oligomer shown in SEQ ID NO:5 with Xba I restriction site were synthesized.

D32

5'- GCGGATCCCGAGCTGCTGGAAGCAGGCTCAG-3'SEQ ID NO:4

5'- CCTCTAGACGGCCGTCGCACTCATTTA-3'SEQ ID NO:5

Please replace the paragraph bridging Pages 36 and 37 with the following:

The DNA fragment coding for the extracellular portion of integrin α4 was obtained by cloning based on reported cDNA sequence information (Takada, Y. et al., EMBO J., 8, 1361-1368 (1989)). The restriction site EcoR I of 1801-base-position of SEQ ID NO:1, the restriction site Stu I of 112-base-position and the restriction site BamH I of 2949-base-position were used for linking the region from the N terminus translation initiation site to Stu I cut site as α4-1, the region from Stu I cut site to EcoR I cut site as α4-2, and the region from EcoR I detached site to BamH I detached site as α4-3. The detailed methods are described below.

D33

Please replace the paragraph bridging Pages 37 and 38 with the following:

The portion coding for α4-1 was designed to be cloned by linking the DNA oligomers of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9, and the DNA oligomers shown in SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9 were synthesized. For the SEQ ID NO:6 and SEQ ID NO:7, restriction site Xba I was added on the side to code for the N terminus, for linking to a vector. Furthermore, compared with the known sequence information, the bases at the 60-, 63- and 64-positions were substituted from C to T, C to A and C to G respectively, and the bases at the 112- and 114-positions were substituted from C to A and C to G respectively. Because of substitution at the 112- and 114-position, restriction site Stu I

D34

was inserted on the side to code for the N termini of SEQ ID NO:8 and SEQ ID NO:9. The 5' termini of the synthesized oligomers were phosphated and annealed, and were linked using T4DNA ligase. After completion of linking, restriction enzymes Xba I and Stu I were used for cutting, and electrophoresis was effected by 5% agarose (NuSieve GTGagarose, FMC) gel. The intended DNA fragment (α4-1) of about 120 bp was cut out and purified.

5'-CTAGACCACCATGTTCCCCACCGAGACGCATGGCTTGGGAAGCGAGGCGCGAACCCGGGCCC CGGAGCTGCA-3'SEQ ID NO:6

5'-GCTTCGGGGCCCGGGTTCGCGCCTCGCTTCCCAAGCCATGCGCTCTCGGTGGGGAAC ATGGTGGT-3'SEQ ID NO:7

D34

5'-CTCCGGGAGACGGTGATGCTGTTGCTGTGCCTGGGGGTCCCGACCGGCAGG-3'SEQ ID NO:8

5'-CCTGCCGGTCGGGACCCCCAGGCACAGCAACAGCATCACCGTCTCCCGGAGTCGA-3'SEQ ID NO:9

Please replace the first full paragraph on Page 38 with the following:

Then, the RNA of human osteosarcoma cell line MG63 (ATCC CRL 1427) as an integrin α4 expressing cell was separated, and PolyA(+)RNA was purified using oligo dT cellulose column (NEB). Based on it, a single stranded cDNA was synthesized using a reverse transcriptase (GIBCO), for use as the template for PCR. As primers for amplifying α4-2 and α4-3 DNAs, four DNA oligomers of SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:13 with Pst I and Stu I restriction sites inserted (SEQ ID NO:10) or BamH I

D35

restriction site inserted (SEQ ID NO:13) were synthesized.

5'-CACTGCAGGCAGGCCTTACAACGTGGACACTGAGAGC-3'SEQ ID NO:10

D35 Cont

5'-GCAGAAACCTGTAAATCAGCAG-3'SEQ ID NO:11

5'-GCATTTATGCGGAAAGATGTGC-3'SEQ ID NO:12

5'-CGGGATCCGTGAAATAACGTTTGGGTCTT-3'SEQ ID NO:13

Please replace the first full paragraph on Page 39 with the following:

The α4-1-2 Bluescript was digested by restriction enzyme Not I, and blunted at the termini by T4DNA polymerase treatment, being digested by restriction enzyme EcoR I, to prepare a small DNA fragment. The α4-3 Bluescript was digested by restriction enzymes EcoR I and BamH I, to prepare a small DNA fragment. The two small DNA fragments were simultaneously linked to a large DNA fragment

D36

obtained by digesting $IgG_1SR\alpha$ by restriction enzymes EcoR V and BamH I, to obtain a plasmid DNA. The obtained base sequence coding for integrin α4•IgG heavy chain chimeric protein is shown as SEQ ID NO:1. The plasmid (integrin α4•IgGSRα) is hereinafter called integrin α4•IgG heavy chain chimeric protein expression vector.

Please replace the paragraph bridging Pages 39 and 40 with the following:

D37

The RNA of human fibroblast line MRC5 (ATCC CCL 171) as an integrin β1 expressing cell was separated, and oligo dT cellulose column was used to purify PolyA (+)RNA. Based on it, a single stranded cDNA was synthesized using a reverse transcriptase, and was used as the template for PCR. As primers, two DNA oligomers of

SEQ ID NO:14 and SEQ ID NO:15 with BamH I site (SEQ ID NO:15) inserted on the side coding for C terminus were synthesized according to the sequence information (Cott Argraves, W. et al., J. Cell Biol., 105, 1183-1190 (1987)).

5'-GCGGAAAAGATGAATTTACAAC-3'(SEQ ID NO:14)

5'-GTGGGATCCTCTGGACCAGTGGGACAC-3'(SEQ ID NO:15)

Please replace the first full paragraph on Page 40 with the following:

The template cDNA, primers, dNTPs and Taq polymerase were mixed in a PCR buffer, and treated by a thermal cycler at 94°C for 1 minute for DNA denaturation, at 57°C for 2 minutes for annealing the primers and at 72°C for 3 minutes for elongating the primers. This treatment was performed 30 cycles. The amplified DNA was smoothened at the termini by T4DNA polymerase treatment, and digested by restriction enzyme BamH I. Then, the DNA fragment was purified. Subsequently, the DNA fragment obtained in PCR before was sub-cloned at the Sma I and BamH I sites of pBluescriptKS(+). A small DNA fragment purified by digesting it by restriction enzymes EcoR I and BamH I was inserted into a large DNA fragment of $IgG_1SR\alpha$ treated by restriction enzymes EcoR I and BamH I, to obtain a plasmid DNA. The obtained base sequence coding for β1•IgG heavy chain chimeric protein is shown in SEQ ID NO:2. The plasmid (integrin β1•IgGSRα) is hereinafter called integrin β1•IgG heavy chain chimeric protein expression vector.

Please replace the paragraph bridging Pages 42 and 43 with the following:

Fifty microliter per well of anti-human integrin α4 antibody (Becton & Dickinson, Clone L25.3) or anti-human integrin β1 antibody (Coulter, Clone 4B4) (12μg/ml each) was put into a 96-well immunoplate (NUNC), and allowed to stand at 4°C for 16 hours. Then, each well was washed by Dulbecco's phosphate buffered saline (Nissui Seiyaku, not containing Ca or Mg ions, hereinafter called PBS(-)) twice, and non-specific reaction was blocked by PBS(-) containing 25% Block Ace (Snow Brand Milk Products Co., Ltd.). After blocking, the culture supernatant solution of CHO cells grown in selective medium was properly diluted, and reacted with the coated antibody at room temperature for 1 hour. After reaction, the surface of the plate was washed with 0.02% Tween-containing PBS(-) (hereinafter called T-PBS) twice. It was then caused to react with biotinylated anti-human IgG antibody (Vector) for 1 hour, and the reaction mixture was washed with T-PBS twice, and in succession caused to react with avidin-horseradish peroxidase (Sigma) for 1 hour. The reaction mixture was washed with PBS(-) twice. The PBS(-) was perfectly aspirated, and orthophenylenediamine was used as a substrate for color development. The absorbance values at 490 nm were measured using a microplate reader (Bio-rad NOVAPATH), and the clone showing a high absorbance value was selected.

D39

Please replace the first full paragraph on Page 44 with the following:

The CHO cells highly capable of producing the α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex were cultured in nucleic acid-free αMEM medium containing 5% FBS (Ultra-low IgG grade, GIBCO) (hereinafter called αMEM(-) medium, GIBCO BRL) for one day, to reach semiconfluence, and they were cultured in αMEM(-) medium containing 1% FBS (Ultra-low IgG grade) for 3 days, and the culture supernatant was collected. It was concentrated to 1/10 volume by ultrafiltration using Prep-scale (Millipore), and 1M Hepes solution (pH 8.0) was added to achieve a final concentration of 5 mM, for preparing a starting solution for further purification.

Please replace the first paragraph on Page 45 with the following:

FMP activated Cellulofine (Seikagaku Kogyo) was equilibrated by a coupling buffer (50 mM Na_2CO_3-$NaHCO_3$ pH 8.5), and a peptide showing SEQ ID NO:3 (hereinafter called CS-1 peptide) synthesized by a peptide synthesizer was added. The mixture was inverted and mixed at 4°C for 16 hours.

Cys Leu His Gly Pro Glu Ile Leu Asp Val Pro Ser Thr SEQ ID NO:3

After completion of mixing, the mixture was washed with the coupling buffer, and a blocking buffer (0.1 mM monoethanolamine, 50 mM Tris-HCl, pH 8.0) was added. The mixture was inverted and mixed

further at room temperature for 6 hours. Then, the mixture was sufficiently washed with TBS solution (150 mM NaCl, 20 mM Tris-HCl, 1 mM $MnCl_2$, pH 7.5), to prepare CS-1 peptide bound Cellulofine column. To the column the starting solution for further purification was applied and allowed to stand at room temperature for 3 hours, and washed with 10 times the column volume of a washing buffer (1M NaCl, 0.1% Triton, 20 mM Tris-HCl, 1 mM $MnCl_2$, pH 7.5) and the same volume of the TBS solution. After completion of washing, an elution buffer (10 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5) was used to elute the proteins bound to the CS-1 column. The eluate was collected and dialyzed against PBS(-).

D41 Cont

Please replace the paragraph bridging Pages 45 and 46 with the following:

The eluted fractions of (3) were subjected to SDS-PAGE under non-reducing or reducing conditions using 6.0 or 7.0% acrylamide gel, and the gel was stained with Coomassie-blue. As a result, under non-reducing conditions, two bands considered to be attributable to the α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex and its polymer were observed. Under reducing conditions, two bands (170 kDa and 135 kDa) considered to be attributable to the integrin α4•IgG heavy chain chimeric protein and the integrin β1•IgG heavy chain chimeric protein and two bands (80 kDa and 90 kDa) considered to be attributable to the intramolecular cleavage of the integrin α4•IgG heavy chain chimeric protein (Hemler, M. E. et al., J. Biol. Chem., 262, 11478-11485

D42

(1987)) were observed. These results suggest that the eluted protein of (3) has a molecular structure considered to be α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex, and that the molecules constituting the heterodimer are linked by a disulfide bond between the IgG heavy chains.

D42 cont

Please replace the paragraph bridging Pages 46 and 47 with the following:

The basic method conformed to a published book ("Antibodies", Harlow, E. et al., (1988), Cold Spring Harbor Lab. Press, New York). That is, the eluted protein of Example 6 (3) considered to be α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex was ^{125}I-labeled using the lactoperoxidase method. Then, Affigel-10 (Bio-rad) was washed with 0.1 M Hepes solution (pH 8.0), and normal murine IgG, anti-human integrin α4 antibody (clone 11C2B) and anti-human integrin β1 antibody (clone 4B4) were added. Reaction was effected at 4°C for 16 hours to cause covalent bonding, to prepare normal murine IgG beads and the respective antibody beads. Then, the ^{125}I labeled α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex and normal murine IgG beads were inverted and mixed at 4°C for 4 hours for preclearing, and the mixture and the antibody beads were inverted and mixed at 4°C for 16 hours. After completion of mixing, the beads were washed with a washing buffer (200 mM Tris-HCl, 0.5 M NaCl, 0.1% NP-40, 1 mM $MgCl_2$ or 10 mM EDTA, pH 8.0) three times. After completion of washing, a sample buffer for

D43

electrophoresis was added to the beads for treatment at 100°C for 5 minutes, and the mixture was centrifuged. The supernatant solution was analysed by electrophoresis under reducing conditions. After completion of electrophoresis, the gel was dried by a gel dryer, and the protein was detected by autoradiography.

Please replace the first paragraph on Page 48 with the following:

On the other hand, the immunoprecipitation pattern obtained by using the anti-integrin β1 antibody beads in the presence of 10 mM EDTA was the same as that in the presence of 1 mM $MgCl_2$, to clarify that the association between integrin α4•IgG heavy chain chimeric protein and integrin β1•IgG heavy chain chimeric protein does not depend on cations. The above results suggest that the eluted protein obtained in (3) of Example 6 was certain α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex, and if the result of (4) of Example 6 is also taken into account, it is strongly suggested that the association between both the proteins is stable association through a disulfide bond existing between the IgG heavy chains.

Please replace the paragraph bridging Pages 49 and 50 with the following:

The binding of α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex produced by CHO cells to the ligand of integrin α4β1 was examined by using the cells expressing VCAM-1. Human normal umbilical intravenous endothelial cells were cultured

with IL-1 3U/ml for 16 hours, to prepare VCAM-1 expressing cells. The cells were treated by 1 mM EDTA at 37°C for 15 minutes, for dispersion as single cells. The cells (2×10^5 cells per sample tube) were cultured with the supernatant of the CHO cells producing α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex for 30 minutes in the presence of 1 mM (final concentration) $MnCl_2$ or 3 mM (final concentration) EDTA. After completion of reaction, the cells were washed twice by centrifugation at 1200 rpm at room temperature for 5 minutes using a buffer for binding assay (24 mM Tris-HCl, 10 mM Hepes, 150 mM NaCl, 1 mM $MnCl_2$or 1 mM EDTA, 1% BSA, 2 mM glucose, pH 7.4). After washing, FITC labeled anti-human IgG antibody (Cappel) was added, and incubated at room temperature for 20 minutes. The cells were washed by the same buffer, and the chimeric proteins bound to the cells were determined by a flow cytometer (ELITE, Coulter).

D45 Cont

Please replace the third full paragraph on Page 51 with the following:

D46

The capability of α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex to be bound to the peptide fragment SEQ ID NO:3 of the other ligand, fibronectin was also examined.

Please replace the fourth full paragraph on Page 51 with the following:

D47

At first, according to one report (Humphries, M. J. et al., J. Biol. Chem., 262, 6886-6892 (1987)), the peptide fragment of

SEQ ID NO:3 (CS-1 peptide) was bound to rabbit IgG (Sigma), to prepare CS-1-IgG. The CS-1-IgG was diluted by PBS(-), and put in a 96-well immunoplate (NUNC) by 100 µl/well, and allowed to stand at 4°C for 16 hours, to be coated on the plate.

Please replace the paragraph bridging Pages 51 and 52 with the following:

After completion of standing, the surface of the plate was washed with PBS(-) twice and treated with denatured 1% BSA (heat-denatured at 80°C for 10 minutes)-PBS solution (300 µl/well)at 4°C for 3 hours, to block the nonspecific reaction. Then, the solid phase CS-1-IgG and the CHO culture supernatant (100 µl) containing α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex were reacted with each other at 30°C for 3 hours. The non-bound α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex was removed by washing with 0.1% BSA-containing TBS buffer (150 mM NaCl, 25 mM Tris-HCl, 1 mM $MnCl_2$, pH 7.4) twice, and the bound α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex was detected by biotin labeled anti-human IgG antibody (Vector) as the primary antibody and avidin labeled horseradish peroxidase (Sigma) as the secondary antibody. The surface of the plate was washed with the TBS buffer. Orthophenylenediamine was added as a substrate for color development, and the absorbance values at 490 nm were measured.

Please replace the second full paragraph on Page 53 with the following:

In the binding determination system of Example 9, the effects of three peptides, i.e., SEQ ID NO:16 (hereinafter called GPEILDVPST), SEQ ID NO: 17(hereinafter called GPEILEVPST) and SEQ ID NO:18 (hereinafter called GRGDSP) were examined.

Gly Pro Glu Ile Leu Asp Val Pro Ser Thr SEQ ID NO:16

Gly Pro Glu Ile Leu Glu Val Pro Ser Thr SEQ ID NO:17

Gly Arg Gly Asp Ser Pro SEQ ID NO:18

Please replace the paragraph bridging Pages 53 and 54 with the following:

The peptides were synthesized by a peptide synthesizer. The peptide and 100 μl of CHO cultured supernatant solution containing α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex were mixed at room temperature for 20 minutes, and the binding to CS-1-IgG was determined according to the method of Example 9. The results are shown in Fig. 3. GPEILDVPST (SEQ ID NO:16)showed temperature-dependent inhibitory activity in a range of 0.1 to 10 μmg/ml, but GPEILEVPST (SEQ ID NO:17) and GRGDSP (SEQ ID NO:18) did not show any inhibition of binding. These results show that the binding determination system of Example 9 allows detection of the inhibitory effect of the peptide (GPEILDVPST) (SEQ ID NO:16)inhibiting the binding between integrin α4β1 and CS-1 peptide specifically.

Please replace the first full paragraph on Page 54 with the following:

The DNA fragment coding for the extracellular portion of integrin α2 was divided into α2-1 and α2-2 based on the reported cDNA sequence information (Takada, Y. et al., J. Cell. Biol., 109, 397-407 (1989)) and subcloned, and they were integrated on an expression vector. At first, the RNA of human fibroblast line MRC-5 (ATCC CCL 171) as integrin α2 expressing cell was separated, and an oligo dT cellulose column was used to purify PolyA(+)RNA. Based on it, a single stranded cDNA was synthesized and used as the template of PCR. As PCR primers, DNA oligomers of SEQ ID NO:20 and SEQ ID NO:21 were synthesized for α2-1, and DNA oligomers of SEQ ID NO:22 and SEQ ID NO:23, for α2-2.

D51

5'-GCTCGAGCAAACCCAGCGCAACTACGG-3' SEQ ID NO:20

5'-ATAGTGCCCTGATGACCATTG-3' SEQ ID NO:21

5'-GATGGCTTTAATGATGTGATTG-3' SEQ ID NO:22

5'-TGTTGGTACTTCGGCTTTCTC-3' SEQ ID NO:23

Please replace the paragraph bridging Pages 54 and 55 with the following:

The template cDNA, primers, dNTPs and Taq polymerase were mixed in a PCR buffer and PCR was performed 30 cycles by a thermal cycler (reaction conditions: 94°C 1 minute - 60°C 2 minutes - 72°C 3 minutes). The amplified DNA fragment of α2-1 was digested by restriction enzymes Xhol and EcoR1, and the DNA fragment of α2-2 was blunted at the termini by T4DNA polymerase treatment and digested by restriction enzyme EcoR I. Each fragment was purified. The two purified DNA fragments were caused to react in a

D52

phosphating reaction solution (50 mM Tris-HCl, 10 mM $MgCl_2$, 25 mM DTT, 1 mM ATP, 0.1 U/μl T4 polynucleotide kinase (Takara), pH 8.0) at 37°C for 1 hour, and the reaction mixture was heat-treated at 68°C for 5 minutes to inactivate the enzyme. Then, $IgG_1SR\alpha$ prepared in Example 1 was digested by restriction enzyme BamH I and caused to react in Klenow reaction solution (66 mM Tris-HCl, 10 mM , $MgCl_2$ 10 mM DTT, 0.2 mM dNTPs, 0.05 U/μl Klenow fragment (Takara), pH 7.5) at 37°C for 30 minutes, to blunt the termini, and the reaction mixture was heat-treated at 70°C for 5 minutes to inactivate the enzyme. Furthermore, a large DNA fragment was digested by restriction enzyme Xho I, and purified. The two (α2-1 and α2-2) DNA fragments phosphated before were inserted into the large DNA fragment, to obtain a plasmid DNA. The obtained base sequence coding for integrin α2•IgG heavy chain chimeric protein is shown in SEQ ID NO:19. This plasmid (integrin α2•IgGSRα) is hereinafter called α2•IgG heavy chain chimeric protein expression vector.

Please replace first full paragraph on page 57 with the following:

Fifty microliter per well of anti-human integrin α2 antibody (Becton & Dickinson, clone P1E6) or anti-human integrin β1 antibody (clone 4B4) (2 μg/ml each) was put into a 96-well immunoplate, and allowed to stand at 4°C for 16 hours. Then, each well was washed with PBS(-) twice, blocked and the culture supernatant of the CHO cells grown in second selective medium was properly diluted and reacted with the coated-antibody at room temperature for 1 hour.

After the reaction, the surface of the plate was washed with T-PBS twice, and caused to react with biotinylated anti-human IgG antibody for 1 hour and with avidin-horseradish peroxidase for 1 hour, and the reaction mixture was washed with PBS(-) twice. After completion of reaction, orthophenylenediamine was used as a substrate for color development, and the absorbance values at 490 nm were measured using a microplate reader. A clone showing a high absorbance value was selected.

D53 Cont

Please replace the first paragraph on Page 58 with the following:

The CHO cells highly capable of producing α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex were cultured in an αMEM(-) medium containing 5% FBS (Ultra-low IgG grade) for 1 day, to reach semiconfluence, and they were cultured on an αMEM(-) medium containing 1%FBS (Ultra-low IgG grade) for 3 days. The culture supernatant was collected, and concentrated to 1/10 volume by ultrafiltration. Then, 1M Hepes solution (pH 8.0) was added to achieve a final concentration of 5 mM, to obtain a starting solution for further purification.

D54

(2) Protein A column chromatography

Please replace the last paragraph on page 59 with the following:

The eluted fraction of (3) was subjected to SDS-PAGE using 7.0% acrylamide gel under non-reducing or under reducing conditions, and the gel was stained with Coomassie-blue. As a

D55

result, a band considered to be attributable to α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex was observed. Under reducing conditions, two bands (185 kDa and 135 kDa) considered to be attributable to integrin α2•IgG heavy chain chimeric protein and integrin β1•IgG heavy chain chimeric protein were observed. These results suggest that the eluted protein has a molecular structure considered to be α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex, and is linked by a disulfide bond between the IgG heavy chains.

D55 cont

Please replace the first paragraph on Page 60 with the following:

The eluted protein of (3) of Example 14 was ^{125}I-labeled, and subjected to immunoprecipitation using the beads coupled with normal murine IgG, anti-human integrin α2 antibody (clone P1E6) and anti-human integrin β1 antibody (clone 4B4) as described in Example 7, and to SDS-PAGE/autoradiography under reducing conditions.

D56

Please replace the second paragraph on Page 60 with the following:

As a result, in both 1 mM $MgCl_2$ and 10 mM EDTA, from the beads of both anti-human integrin α2 antibody and anti-human integrin β1 antibody, the same precipitation patterns expected from the structure of α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex could be obtained. These results show that the eluted protein obtained in (3) of Example 14• is certainly α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer

D57

complex, and with the results of (4) of Example 14 also taken into account, it is strongly suggested that the association of both the proteins is stable through a disulfide bond existing between the IgG heavy chains.

D57 cont

Please replace the second paragraph on Page 61 with the following:

At first, a collagen (Cell Matrix Typel 3 mg/ml) was diluted to 0.1 μg/ml by 0.02M acetic acid solution, and put in an immunoplate by 100 μl/well, being kept at 4°C for 16 hours. Then, the collagen solution was removed by suction, and the plate was washed with PBS(-) twice for neutralization. Heat- denatured 1% BSA-PBS solution was put in the plate by 300 μl/well for blocking at room temperature for 3 hours. After completion of blocking, it was rinsed with PBS(-) twice, to prepare a collagen coated plate.

D58

Please replace the paragraph bridging Pages 62 and 63 with the following:

At first, α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex purified in Example 6, or human IgG was prepared at a proper concentration by PBS(-) and was coated on a plastic plate at 4°C for 16 hours, being coated on a plastic plate. Then, according to a report (Cott, J. K. and Smoth, G. P., Science, 249, 386-390 (1990)), a phage peptide library in which a random six amino acid residues were cyclyzed by the disulfide bond of cysteine at both the ends was prepared and suspended in 0.1% BSA-containing TBS buffer. The phage peptide library was reacted with human IgG

D59

at 30°C for 3 hours, to absorb phage peptides capable of being bound to IgG. Then, the non-absorbed phases were reacted with α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex at 30°C for 3 hours, and the reaction mixture was washed with 0.1% BSA-containing TBS buffer twice to remove the phage peptides incapable of being bound to the heterodimer complex. Only the phage peptides capable of being bound were collected after elution with 0.1M glycine-hydrochloric acid (pH 2.2). After collection, the phage was amplified and the above mentioned binding operation was repeated further twice. Only the phage peptides capable of being bound to the heterodimer complex were selectively concentrated. In the final elution operation, phage peptides capable of being bound to the heterodimer complex were eluted using 10 mM EDTA and 0.1M glycine-hydrochloric acid in two steps, and the amino acid sequences of the respective peptides were analyzed. Of them, eight sequences (SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30 and SEQ ID NO:31) are shown in Table 1. Furthermore, they were examined using the binding assay system of Example 9, and the IC50 values of the four peptide sequences showing binding inhibitory activity are shown in Table 1.

D 59 Cont

In Table 2, fourth column, please replace the following:

Sequence No.	with	SEQ ID NO:

Please replace the title of Example 18 on Page 64 with the following:

Example 18

Acquisition of a low molecular weight compound capable of inhibiting the binding between the peptide fragment on fibronectin and α4•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex

D60

Please replace the first full paragraph on Page 66 with the following:

The amount of α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex bound on the liposome was determined by a densitometer (ATTO) after SDS-PAGE/ Coomassie staining, and adjusted to final concentration of 1 mg/ml.

D61

Please replace the second full paragraph on Page 67 with the following:

A collagen (Cell Matrix Typel, 3 mg/ml) was diluted by 0.02M acetic acid solution, and the solution was put in an immunoplate by 100 μl/well, being kept at 4°C for 16 hours. Then, the collagen solution was removed by suction, and the plate was washed with PBS(-) twice for neutralization, and heat- denatured 1% BSA-PBS solution was put in the plate by 300 μl/well for blocking at room temperature for 3 hours. After completion of blocking, the plate was rinsed with PBS(-) twice, to prepare a collagen coated plate.

D62

Please replace the paragraph bridging Pages 67 to 69 with the following:

Normal human plasma (George King) and von Willebrand's factor deficient (severe) plasma (George Kind) were treated for absorption with anti-human IgG antibody and protein A, and dialyzed against PBS(-) for 24 hours, to remove the contained sodium citrate. In order that the Ca ion and Mg ion concentration might be a physiological cation concentration in the blood when used, $CaCl^2$ and $MgCl_2$ were added to achieve final concentrations of 1.2 mM and 0.2 mM respectively. Into the normal human plasma and von Willebrand's factor deficient plasma adjusted in cation concentration, α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex liposome or human IgG liposome was suspended to achieve protein concentrations of 1 to 100 ng/ml. Any of the suspensions was put in the collagen coated plate by 100 µl/well. The plate was shaken by a plate shaker at 100 rpm, for reaction at room temperature for 15 minutes. After completion of reaction, the non-bound liposome was removed by washing with a PB solution (1.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 1% BSA-containing PBS, pH 7.4), and the bound liposome was immobilized by 1% glutaraldehyde-PBS at room temperature for 30 minutes. After completion of immobilization, a heat- denatured BSA-PBS solution was used for blocking at room temperature for 1 hour. Then, as described in Example 16, it was caused to react with biotin labeled human IgG antibody used as a primary antibody and avidin labeled horseradish

peroxidase used as a secondary antibody, and washed with a TBS buffer. Into it, orthophenylenediamine was added as a substrate for color development, and the absorbance values at 490 nm were measured. To examine the effect of 5 mM EDTA, anti-integrin α2 antibody (clone P1E6, 10 μg/ml) and anti-integrin β1 antibody(clone 4B4, 10 μg/ml), it was caused to react with the liposome suspension at room temperature for 15 minutes before reaction with the collagen.

D63 Cont

Please replace the first full paragraph on Page 69 with the following:

The results are shown in Figs. 5 and 6. In the normal human plasma, the human IgG liposome as a negative control was not found to be bound to the collagen, but the binding of α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex liposome to the collagen was increased in a concentration dependent manner. Also when the von Willebrand's factor deficient plasma was used, equivalent binding was detected. Furthermore, the binding to the collagen observed when 30 ng/ml of α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex liposome was added to the normal plasma was completely inhibited by adding EDTA as a cation chelating agent or the antibodies. The results show that in plasma with a physiological cation concentration, α2•IgG heavy chain-β1•IgG heavy chain chimeric protein heterodimer complex liposome is bound to a collagen like platelets, strongly suggesting that it can be a substitute of adhesive platelets and

D64

a reagent for monitoring the collagen exposed region. Furthermore, it is indicated that since equivalent binding activity was shown also in von Willebrand's factor deficient plasma, the liposome can also be used in the plasma with coagulation abnormality such as von Willebrand's disease.

D64 Amt

On page 72, please replace the title of the second paragraph with the following:

SEQ ID NO: 1

Length of sequence: 4228

D65

Type of sequence: Nucleic acid

Sequence

On page 80, please replace the title of the fifth paragraph with the following:

SEQ ID NO: 2

Length of sequence: 3463

D66

Type of sequence: Nucleic acid

Sequence

On page 87, please replace the title of the third paragraph with the following:

SEQ ID NO: 3

D67

Length of sequence: 13

Type of sequence: Amino acid

Topology: Linear

D67 cont

Kind of sequence: Peptide

Sequence

On page 87, please replace th title of the fourth paragraph with the following:

SEQ ID NO: 4

D68

Length of sequence: 31

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Paragraph heading bridging pages 87 and 88, please replace the title with the following:

SEQ ID NO: 5

D69

Length of sequence: 27

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

sequence

On Page 88, please replace the title of the third paragraph with the following:

NE

SEQ ID NO: 6

Length of sequence: 73

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 88, please replace the title of the fourth paragraph with the following:

SEQ ID NO: 7

Length of sequence: 65

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Paragraph heading bridging Pages 88 and 89, please replace the title with the following:

SEQ ID NO: 8

Length of sequence: 51

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 89, please replace the title of the second paragraph with the following:

SEQ ID NO: 9

Length of sequence: 55

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Straight chain

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 89, please replace the title of the third paragraph with the following:

SEQ ID NO: 10

Length of sequence: 37

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 89, please replace the title of the fourth paragraph with the following:

SEQ ID NO: 11

Length of sequence: 22

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 90, please replace the title of the first paragraph with the following:

SEQ ID NO: 12

Length of sequence: 22

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 90, please replace the title of the second paragraph with the following:

SEQ ID NO: 13

Length of sequence: 29

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 90, please replace the title of the third paragraph with the following:

SEQ ID NO: 14

Length of sequence: 22

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Paragraph heading bridging Pages 90 and 91, please replace the title with the following:

SEQ ID NO: 15

Length of sequence: 27

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 91, please replace the title of the second paragraph with the following:

SEQ ID NO: 16

Length of sequence: 10

Type of sequence: Amino acid

Topology: Linear

Kind of sequence: Peptide

Sequence

Page 91, please replace the title of the third paragraph with the following:

SEQ ID NO: 17

Length of sequence: 10

Type of sequence: Amino acid

Topology: Linear

Kind of sequence: Peptide

Sequence

Paragraph heading bridging Pages 91 and 92, please replace the title with the following:

SEQ ID NO: 18

Length of sequence: 6

Type of sequence: Amino acid

Topology: Linear

Kind of sequence: Peptide

Sequence

Page 92, please replace the title of the third paragraph with the following:

SEQ ID NO: 19

Length of sequence: 4675

Type of sequence: Nucleic acid

Sequence

Page 101, please replace the title of the fifth paragraph with the following:

SEQ ID NO: 20

Length of sequence: 27

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 101, please r place the title of the sixth paragraph with the following:

SEQ ID NO: 21

Length of sequence: 21

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 102, please replace the title of the first paragraph with the following:

SEQ ID NO: 22

Length of sequence: 22

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 102, please replace the title of the second paragraph with the following:

SEQ ID NO: 23

Length of sequence: 21

Type of sequence: Nucleic acid

Number of strands: Single

Topology: Linear

Kind of sequence: Other nucleic acid, synthetic DNA

Sequence

Page 102, please replace the title of the third paragraph with the following:

SEQ ID NO: 24

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Paragraph heading bridging Pages 102 and 103, please replace the title with the following:

SEQ ID NO:25

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Page 103, please replace the title of the second paragraph with the following:

SEQ ID NO:26

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Page 103, please replace the title of the third paragraph with the following:

SEQ ID NO:27

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Page 103, please replace the title of the fourth paragraph with the following:

SEQ ID NO:28

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Page 104, please replace the title of the second paragraph with the following:

SEQ ID NO:29

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Page 104, please replace the title of the third paragraph with the following:

SEQ ID NO:30

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence

Page 104, please replace the title of the fourth paragraph with the following:

SEQ ID NO:31

Length of sequence: 8

Type of sequence: Amino acid

Topology: Circular

Kind of sequence: Peptide

Sequence